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The polymorphic cell adhesion molecule CD44 is encoded by a complex gene and undergoes extensive alternative splicing. Differential regulation of CD44 alternative splicing has been implicated in human tumorigenesis, and differences in variant isoform expression have been detected in normal versus tumor breast tissue. Our prospective analysis of 99 human breast samples by RT-PCR and Southern blotting reveals that approximately half of the tumor samples express qualitatively more CD44 than their normal counterparts. Our study also reveals that some normal breast specimens express CD44 variant isoforms. A novel CD44 isoform generated by a unique splicing event has been cloned from BT-20 breast tumor cells and may represent a breast cell specific variant. To identify the cis- and trans-acting signals involved in CD44 splicing, newly constructed CD44 minigenes have been transfected into breast cell lines which will be fused to form transient heterokaryons. SR protein splicing factors have been purified and detected in breast cell lines, and their role in CD44 alternative splicing will be examined.

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Nicole M. Rosnick
Principal Investigator's Signature

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INTRODUCTION

The cell adhesion molecule CD44 is a cell surface transmembrane glycoprotein which exhibits a wide range of biological functions. Originally described as a lymphocytic homing receptor, CD44 is capable of directing the migration of recirculating lymphocytes across high endothelial venular membranes of lymph nodes (1-2) . In addition, CD44 is involved in the processes of lymphocyte activation, inflammation, and serves as a receptor for hyaluronan, chondroitin-4-sulfate, and a number of proteoglycans that possess chondroitin sulfate side chains (3-6). Because CD44 is able to bind ligands of the extracellular matrix, it is hypothesized that CD44 may play a role in cell-matrix and cell-cell adhesion. Although CD44 was originally identified in lymphocytic cells, it has since been shown to be expressed in a wide variety of cell types and tissues such as breast, lung, colon, brain, prostate and epidermis (7).

The CD44 gene is approximately 60 kilobases long and encodes a complex transcription unit comprised of at least twenty exons. The heterogeneity of the CD44 protein was originally believed to be due to extensive modification of the protein including O- and N- linked glycosylation, as well as chondroitin sulfate modification. However, genomic and cDNA cloning and sequencing of the CD44 gene revealed that the molecule's diversity is also attributed to the RNA processing mechanism of alternative splicing (8-11). Twelve of the twenty exons are subject to alternative splicing, and ten of these exons (V1-V10) are spliced at a unique extracellular site to generate numerous CD44 isoforms. The two most prevalent isoforms of CD44, CD44H and R, were first identified on cells of hematopoietic and epithelial origin respectively. CD44R represents the simplest alternatively spliced form of CD44 whereby an additional 132 amino acids are spliced into the extracellular domain of the 85-95 kD CD44H molecule to generate a protein 120-150 kD in size (Appendix, Figure 1). CD44 isoforms larger in size than CD44R are classified as "variants" and are comprised of any combination of the ten alternatively spliced variant exons encoding the extracellular domain of the protein as well as the constituitively expressed standard exons.

Alternative splicing of the CD44 gene to produce variant isoforms has been implicated in the processes of tumorigenesis and metastasis. Immunohistochemical and RT-PCR (reverse transcription-polymerase chain reaction) analyses have demonstated that while the CD44R transcript is weakly expressed in some normal epithelium, expression is greatly increased in carcinomas (12-13). A compelling study conducted in rat carcinoma cell lines demonstrated that transfection of a particular CD44 isoform comprised of several variant exons (designated pMeta-1) served to induce metastasis in cells normally unable to metastasize (14). Human homologues of the rat pMeta-1 clone have been identified in tumor specimens, and homologues of the variant CD44 transcripts expressed in the rat mammary carcinoma metastatic cell line 13762NF have been detected in human breast cancer cell lines (15-16). Thus, differential regulation of the alternative splicing of CD44 may play a role in the pathogenesis of breast cancer.

The purpose of this research is to investigate the role of CD44 in human breast cancer. As initially proposed, the first aim of our study is to examine the expression of the alternatively spliced CD44 isoforms in human breast cancer. Our goal is to test the assumption that differences in alternative splicing of the CD44 transcript exist between normal breast and tumor breast tissue. Our initial strategy has involved processing primary and metastatic breast specimens for RNA and then examining CD44 expression by RT-PCR and Southern blot methodology. Preliminary results comparing matched normal and tumor samples from resected breast tissue indicated that tumor tissue exhibits differences in CD44 expression. These differences are both quantitative and qualitative in that tumors express an overall greater amount of CD44 message, and the transcripts are larger in size and contain more variant exons than normal breast tissue.

The second specific aim of our proposal is to investigate the molecular signals and mechanisms

responsible for regulating the alternative splicing of CD44 in human breast cell lines. We would like to determine what is responsible for the differential regulation of CD44 alternative splicing resulting in detectable differences in CD44 expression between normal and breast tumor cases. Upon elucidating the molecular signals involved, we can begin to understand how their regulation plays a role in the invasive potential and metastatic proclivity of human breast cancer cells .

BODY

Specific Aim 1: To examine the expression of alternatively spliced CD44 isoforms in human breast cancer

A) Prospective study of human breast specimens using RT-PCR and Southern blot methodology

The accumulation and processing of breast specimens for RT-PCR analysis has been the main focus of our research during this first year of funding. As described in the grant proposal, based on our preliminary studies, we were interested in embarking on a more structured and comprehensive prospective study. Although we had already determined the composition of a learning set to evaluate, before beginning the study we continued to collect samples in blinded fashion and perform RT-PCR on the RNA isolated from the tissue. We have now obtained a total of 99 breast cancer cases which include 34 sets of matched normal and tumor breast tissue. All breast samples obtained this past year were processed for RNA and subjected to RT-PCR by standard methods as described in our grant proposal. CD44 specific primers which anneal to sequences within the framework, or standard exons, allow for the amplification of all CD44 transcripts present within a sample. As a control, the actin gene was reverse transcribed and amplified. Although we originally intended to utilize the porphobilinogen deaminase (PBGD) gene as a control, we found that the small size of the amplified product (<100 basepairs) hindered our ability to accurately detect it on 1% agarose gels, along with CD44 isoforms. We therefore synthesized primers to the actin gene which amplifies a PCR product of 350 basepairs.

Following amplification, the PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide. All tissue samples exhibited expression of the CD44H isoform, as detected on agarose gels. In a smaller percentage of samples, CD44R and other higher molecular weight variant isoforms could be visualized on gels. Every gel was capillary blotted and then sequentially hybridized with three different CD44 variant exon probes. These three probes are: CD44 variant exons V3-V4-V5 (357 bp), V6-V7 (261 bp) and V8-V9-V10 (397 bp). The probes were isolated by conventional restriction enzyme digestion and gel isolation techniques, and the enhanced chemiluminescence (ECL) detection system (Amersham) was used for labelling the probes and developing the blots following hybridization. Hybridization results were interpreted simply as a positive or negative signal on the autoradiographs. The nylon filters were stripped prior to hybridization with each different probe.

To some extent, our results confirm what we first reported in the preliminary results of our grant proposal. Out of the 34 matched sets of tumor and normal breast tissue, approximately 47% (16/34) exhibit the phenomenon whereby tumor tissue expresses more CD44 message than its normal counterpart. The hybridization signals are stronger in tumor, and the position of the signal on the filter indicates that more CD44 variant isoforms are present (Appendix, Figure 2; lanes 3-6, 11-14, 19-20, 22-23). Thus in roughly half of our samples we can detect qualitative and quantitative differences in the expression of CD44 when comparing normal and tumor breast tissue. Overall, 43% of the tumors examined express isoforms containing variant exons V3 -V10 based on a positive hybridization signal with all three probes. This supports the hypothesis that increased expression of CD44 variants may play a role in the progression of breast cancer.

Of the 99 samples examined to date, RT-PCR and hybridization results indicate that there is no

significant correlation between tumor type and expression of variant exons. Although we have not yet obtained the clinical diagnosis for each specimen examined, the information we have so far gathered does not reveal any obvious correlation or trend regarding CD44 expression. Most significant is our finding that there does not appear to be any one "breast-specific epitope" comprised of variant exons which might serve as a prognostic indicator of breast disease.

This study also revealed that in 17.6% (6/34) of matched sets, the normal tissue appears to express more CD44 than its tumor counterpart. In the same percentage of cases the CD44 isoform profile appears to be equivalent in normal and tumor tissue. Although our interpretation is strictly qualitative and does not rely on any quantitative PCR data, the observation of variant CD44 expression in some normal breast specimens is significant. The Southern blot in Figure 2 (lanes 1,9,11,13,17) is an example of several normal tissues which express high levels of isoforms including variant exons V3-V10. Our results agree with several recent reports in the literature which demonstrate the presence of variant isoforms in normal breast tissue by immunohistochemistry and RT-PCR (17-18). One study demonstrates the expression of all variant exons on the surface of normal myoepithelial cells while the luminal cells are negative. Mackay and colleagues (18) demonstrated high expression of exon V9 in normal mammary gland epithelium by immunohistochemistry. Normal breast tissue is not the only human tissue which appears to express CD44 variant isoforms (18-19). We hope to extend our analyses on normal breast tissue by performing immunohistochemistry with variant exon-specific monoclonal antibodies as originally proposed.

The fact that variant isoform expression in normal breast was not detected in our preliminary study may be attributed to several factors. In the past year we changed our labelling and hybridization technique to ECL to allow us to terminate the use of radioactivity. The ECL reaction may provide more sensitivity and perhaps better detect the expression of variant isoforms in normal breast tissue. Furthermore, in our initial study of matched breast tissue, we did not capillary blot and hybridize every sample. Our determination of variant isoform expression was based on detection of CD44 isoforms on an ethidium bromide stained gel. After analyzing almost 100 samples by RT-PCR followed by hybridization with three different probes, we now conclude that variant isoforms not detectable on gels often become apparent only after hybridization (see Figure 2, lanes 3,5,7,14,17). This finding serves to underscore the importance of analyzing breast samples for variant expression by hybridization following RT-PCR.

As we have tested breast cancer specimens from UPMC and Magee-Women's Hospital we have been blinded to the other known independent prognostic indicators, e.g. progesterone receptor, lymph node status, tumor size, and grade. However it has been apparent that CD44 variant expression in our study (50%) has consistently been less than reports by our European colleagues (85%). We have recently begun collaboration with Dr. David Tarin (Oxford) and have noted that patients in his practice present with larger, higher grade, more agressive tumors than those seen here. The apparent discrepency between the European experience and ours may be explained by our increased capability to screen and detect earlier stage tumors, thus skewing our results to less agressive malignancies. We are now analyzing the two patient cohorts to determine if such differences exist. Furthermore we will test 25 consecutive tumor samples from Dr. Tarin for CD44 expression.

B) Cloning novel isoforms from breast cell lines

As described in the original proposal, RT-PCR analyses of CD44 expression in breast tumors and cell lines have revealed the existence of numerous CD44 isoforms. Based on the molecular weight sizes of many of the isoforms, they represent CD44 molecules that have yet to be identified. One method for identifying these novel isoforms is to clone and sequence them from the PCR reaction. Cloning variant isoforms from breast tumors using a specialized cloning system (CLONEAMP, Gibco BRL) proved to be unsuccessful; however, we recently isolated a novel variant isoform expressed by BT-20

cells using a different cloning kit (Prime PCR Cloner, 5 prime --> 3 Prime).

A report by Bourguignon and colleagues (20) which demonstrated the existence of several novel breast-specific CD44 transcripts prompted us to investigate if such isoforms are produced by the cell lines in our laboratory. We designed PCR primers which would amplify only those CD44 isoforms generated by a unique splicing event which skips variant exons V1 and V2. A novel isoform amplified with these primers from BT-20 cells was gel isolated and cloned. This isoform, based on its size in a 1% agarose gel, suggests that it includes standard exon 5 spliced to variant exon V3 which is spliced to variant exon V10. Variant exons V4-V9 are therefore spliced out. This variant represents an entirely new CD44 isoform, and although it may not represent one of the most highly expressed isoforms produced by BT-20 cells, it may serve as a valuable tool for studying the regulation of splicing in breast cell lines. Sequencing of the clone is currently underway to confirm the exon composition, and further studies will be initiated. The presence of this CD44 isoform in BT-20 breast cells suggests that the alternative splicing machinery is subject to very specific controls not uniform in all cell lines. We would like to investigate if the expression of such an isoform plays a role in the differential ability of breast cell lines to invade and metastasize.

Specific Aim 2: To investigate the molecular mechanisms regulating the alternative splicing of CD44 in human breast cell lines

A) Identification of CD44 splicing signals

Our strategy for identifying the cis- and trans-acting splice signals which mediate alternative splicing of the CD44 gene involves introducing CD44 minigene constructs into breast cell lines. The transfected cells are then fused to form transient heterokaryons, and CD44 splicing activity in the heterokaryons is compared to the unfused parental cells. The minigene constructs include an alkaline phosphatase gene which serves as a reporter to measure splicing activity in transfected cells. We initiated this part of the study by transiently transfecting the constructs into a host of breast cell lines including BT-20, MDA-MB-435s and ZR-75-30 (all obtained from the ATCC). We found that expression of the alkaline phosphatase gene was not detectable in the breast cell lines.

Our positive control plasmid for transfection is the bacterial *lacZ* gene driven by the cytomegalovirus (CMV) immediate early promoter. Transfection of the control plasmid into breast cells did reproducibly exhibit β-galactosidase activity as measured by a colorimetric assay in approximately 5-10% of the cells. This transfection efficiency was evident in cells transfected by either lipofectamine (Gibco BRL) or calcium phosphate protocols. Since our collaborators in Vancouver have demonstrated strong alkaline phosphatase activity using these same CD44 splicing constructs in hematopoietic cells, we suspect that the lack of activity may be due to the efficiency of the promoter on all of these constructs. Whereas all our successful transient transfections in the breast cell lines described have utilized the CMV promoter, the original CD44 minigene constructs are cloned into the pREP9 vector (Invitrogen) which is driven by the Rous Sarcoma Virus (RSV) promoter.

We recently obtained the same splicing constructs cloned into the pCEP4 vector (Invitrogen) which contains the CMV promoter. Transient transfection of these CD44 minigene constructs into breast cell lines BT-20, MDA-MB-435 and ZR-75-1 are currently underway. These three cell lines exhibit different CD44 isoform profiles (Appendix, Figure 3) as determined by RT-PCR of RNA harvested from these cells. Once we are able to demonstrate alkaline phosphatase activity in cell lines transfected with the pCEP4 constructs we will move ahead with our experimental approach involving the production of transient heterokaryons.

B) Analysis of CD44 expression in human breast cell lines

In the past year several breast cell lines not previously examined by our laboratory have been tested for CD44 expression to determine if any would be better suited for identifying CD44 splicing signals. These cell lines include MDA-MB-435s, -261 and -468 cells, as well as Hst-578t, ZR-75-30 and ZR-75-1 cells. RT-PCR has been performed on these cell lines, and it appears that each expresses a different profile of CD44 isoforms. In conjunction with RT-PCR analysis of cell lines, immunofluorescence of cell surface proteins utilizing CD44 antibodies specific for the H and R isoforms and several variant exon-specific monoclonal antibodies have been conducted. We plan to transfect each different cell line to determine which exhibits the highest transfection efficiency, as well as test each line for transient heterokaryon formation. Our rationale for these additional experiments is to determine which cell lines are the best candidates for pursuing our strategy of identifying the signals which regulate alternative splicing of the CD44 gene.

C) Determining the role of SR proteins in CD44 gene expression

As originally described, we would like to utilize the CD44 gene in an in vitro system to identify cis- and trans- acting splicing signals responsible for regulating CD44 gene expression. If variant isoform expression does play a role in the ability of cells to invade and metastasize, we would like to exploit the regulation of the splicing machinery to control potentially tumorigenic cells. While we are preparing to utilize the CD44-alkaline phosphatase minigene constructs in transfection experiments, we have adopted an additional approach to studying splicing factors. We have recently begun to examine the role of the SR proteins in CD44 gene expression. The family of SR proteins is comprised of six essential pre-mRNA splicing factors, 20-75 kD in size, that are evolutionarily conserved (21-23). The primary amino acid sequences are highly conserved and each shares a carboxyl-terminal domain of variable length consisting of alternating serine and arginine residues. One report in the literature demonstrated tissue specific differences in the ratios of these proteins and suggested that these differences could account for alternative splice site selection (23). We are interested in investigating if such a phenomenon could account for differential splicing of the CD44 gene.

Our initial strategy is to purify the proteins from each of our breast tissue cell lines and demonstrate that we can detect the SR proteins by immunoblotting. We have already obtained the monoclonal antibody mAb104 (from the ATCC) which recognizes the entire family of SR proteins, and by following the purification protocol described in the literature, we have successfully detected the SR proteins in MDA-MB-468 cells (Appendix, Figure 4). Subsequent to purifying and identifying the family of SR proteins in cell lines MDA-MB-435s, BT-20, ZR-75-1 and ZR-75-30, we will examine methodologies to compare the ratios of each protein. Densitometry of immunoblots may reveal whether the ratios differ amongst the cell lines. Should we find differences we will then determine if these differences can account for the differential expression of CD44 isoforms. Although this was not originally proposed in the grant, we feel it is a reasonable goal and will complement the original strategy to identify cis-acting splicing signals involved in CD44 expression.

CONCLUSIONS

The results of our ongoing prospective analysis of CD44 gene expression in breast tissue do continue to demonstrate that differences can be detected between normal and tumor breast tissue. It is still our goal to explore IF and HOW these differences actually translate into the invasive and metastatic proclivity of a breast cell. However, pursual of the experimental approaches described in Specific Aim 2 of our proposal may provide us with more definitive answers. We have therefore decided to focus more closely on those experiments.

Great strides have been taken towards completing the retrospective RT-PCR, quantitative RT-PCR and immunohistochemical analyses of human breast specimens to determine expression of CD44. We intend to more closely analyze the demographics of the breast cancer cases so far examined in order to determine if CD44 expression correlates with any measure of metastatic potential. The survivability, lymph node positivity and site of metastasis (if applicable) of each case will be investigated. As we continue to process additional samples, we intend to identify the existence of any "breast specific isoforms," as opposed to investigating any general qualitative differences between normal and tumor breast tissue. Based on our cloning of a novel CD44 variant isoform as described above, we may choose to focus on a particular combination of variant exons and test for their expression not only by RT-PCR, but by immunohistochemistry as well.

There still remains the question of how differences in CD44 expression are achieved, and how alternative splicing is regulated in normal versus tumor cells. If we have any hope of one day exploiting those differences for the purpose of developing effective molecular therapies for breast cancer patients, there is a tremendous need for continuing the investigation of CD44 expression and regulation in breast cancer.

ADDITIONAL APPROACHES

Over the past year we have considered some additional approaches to investigating the role of CD44 expression in human breast cancer. If expression of particular CD44 isoforms confers upon a cell the ability to invade or metastasize, we would like to identify those molecules. We have initiated a series of pilot experiments to first test the invasiveness of breast cell lines in vitro. Breast cells are seeded into Transwell chambers (Costar) containing a microporous polycarbonate membrane. After a 24-72 hour incubation, cells are harvested from both the upper and lower chambers of a cluster dish containing the Transwell insert and counted using a Coulter counter to determine their ability to cross through the membrane. This system can be manipulated to include a basement membrane matrix such as Matrigel (Collaborative Biomedical Products) which is comprised of a host of basement membrane matrix proteins including laminin and collagen IV, as well as growth factors.

Upon demonstrating the ability of certain breast cell lines to invade, we would like to determine if repressed expression of any particular CD44 transcript affects invasion. One strategy for testing a molecule's function is to repress its expression and then study the effect in vivo. We have available in our laboratory a variety of hammerhead ribozyme clones designed to recognize and cleave the CD44 transcript. Each ribozyme was specifically designed to recognize a different portion or exon within the CD44 message, and numerous control ribozymes were synthesized in parallel. Breast cell lines stably transfected with the ribozyme constructs, as well as the appropriate controls, will be tested in invasiveness assays. If we can show that a decrease in CD44 expression correlates with invasive potential, we can then design experiments to further delineate which regions or exons of the CD44 gene encode domains responsible for invasion. Although this approach does not directly address the role of splicing signals in CD44 expression, it may still provide us with more insight into the role of CD44 expression in metastatic breast cancer.

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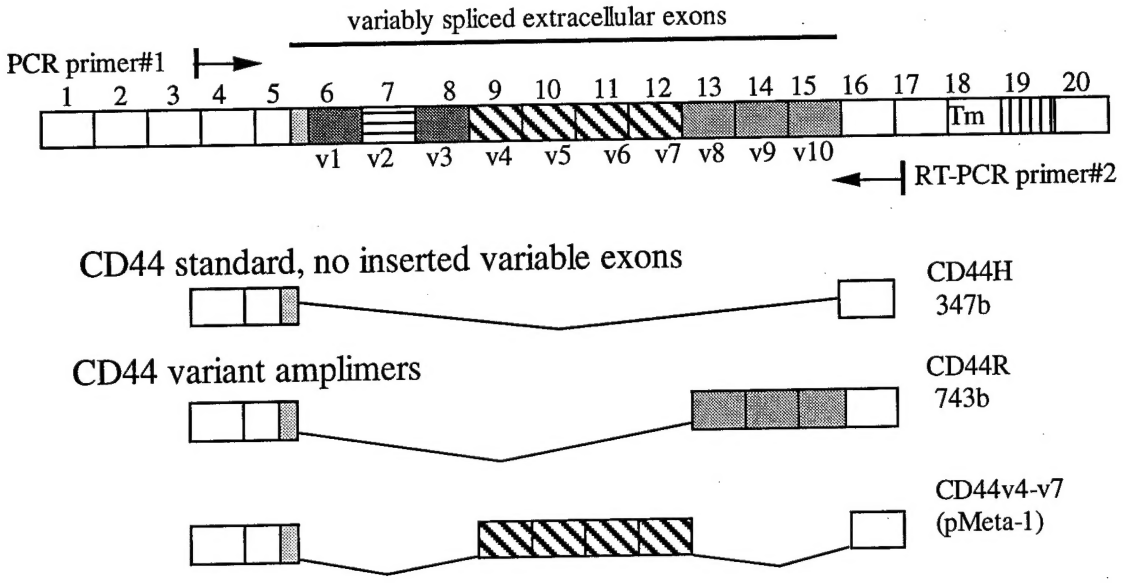

Figure 1: The complex transcription unit of the CD44 gene.

Exons are represented as boxes and are numbered. Numbers along the top denote the conventional exon numbering (1-20) while numbers along the bottom denote the the variant exons (V1-V10) which undergo alternative splicing within the extracellular domain of the molecule. The positions where the two PCR primers used for polymerase chain reaction anneal to CD44 cDNAs are indicated by arrows. Tm signifies transmembrane domain. The two most prevalent CD44 isoforms generatedby alternative splicing, CD44H and CD44R, are depicted, as well as the pMeta-1 isoform described in the text.

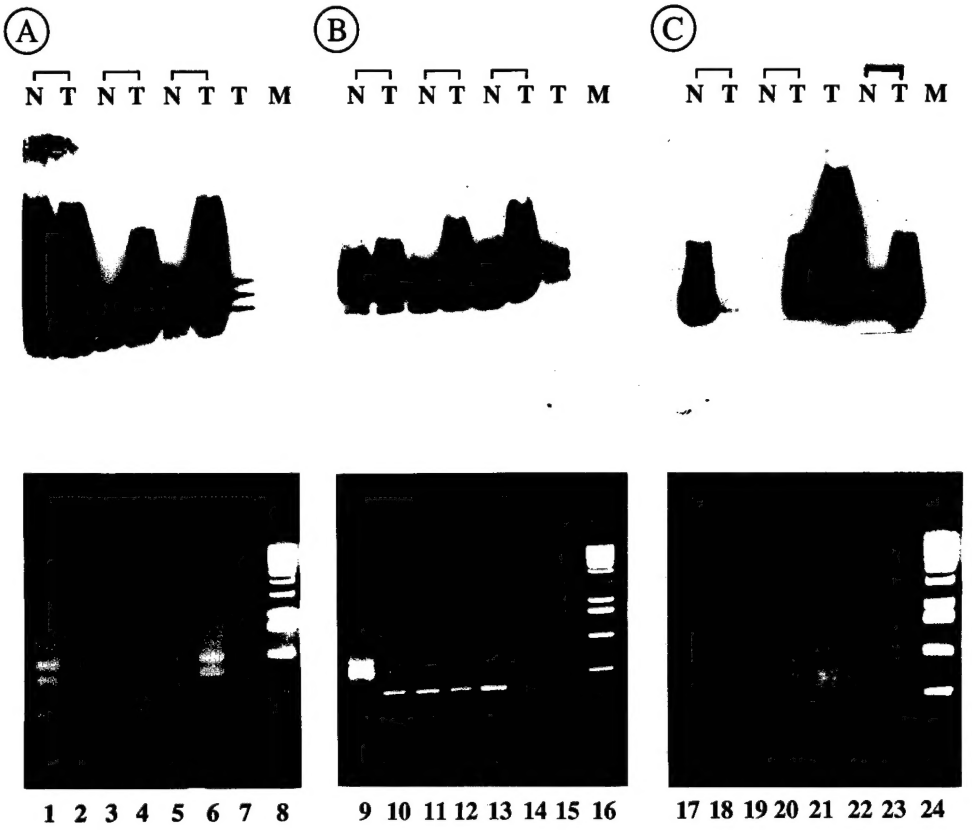

Figure 2: RT-PCR and Southern blot analyses of human breast specimens.

Panels A, B, and C represent a sampling of the results obtained from RT-PCR and Southern blot analysis of human breast tissue. Following RT-PCR of RNA isolated from each tissue specimen using CD44-specific primers, the PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide. The gels were photographed as shown in the lower panels, and then capillary blotted and hybridized with CD44 variant exon-specific probes labelled by the ECL protocol (top panels). Panel A was hybridized with a probe directed against V8-V9-V10, panel B with a probe directed against V6-V7, and panel C with a probe directed against V3-V4-V5. Specimens are labelled as N which denotes normal breast, or T which denotes tumor breast tissue. Brackets indicate matched sets of normal and tumor tissue, and M denotes marker lanes. Each lane represents a different tissue specimen.

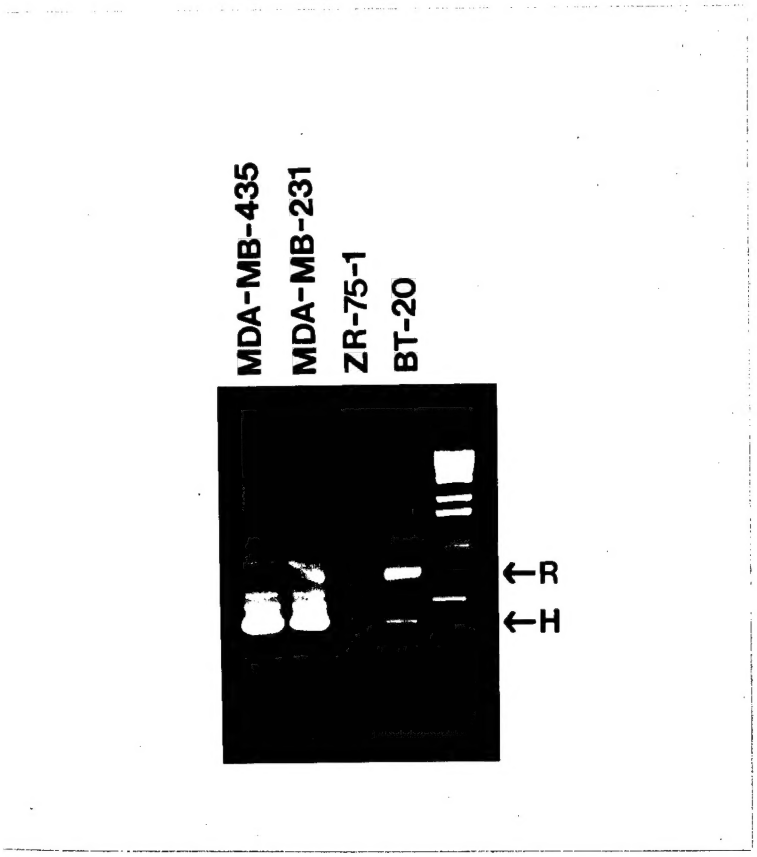

Figure 3: CD44 expression in human breast cell lines.

Cell lines were analyzed for CD44 expression by RT-PCR. CD44H and R isoforms were identified by their sizes on the agarose gel relative to molecular weight markers, and are denoted by arrows. PCR products larger in size than CD44R represent additional variant isoforms.

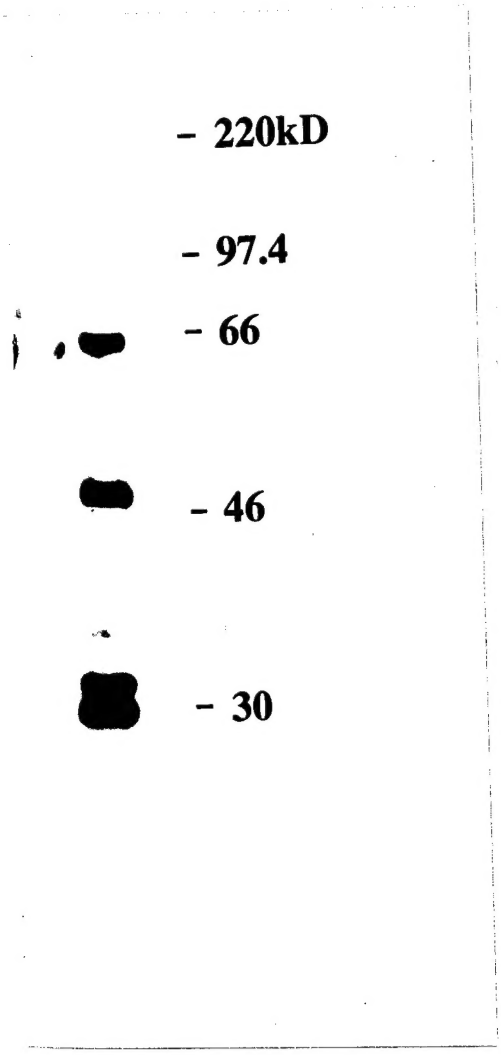

Figure 4: SR proteins can be detected in MDA-MB-468 cells.

The family of pre-mRNA splicing factors, the SR proteins, were purified from MDA-MB-468 breast tumor cells following the published protocol. Purified proteins were run on a 10% acrylamide gel, transferred to a nylon filter and immunoblotted with mAb104 which recognizes the family of SR proteins. Five of the six SR proteins were detected following ECL detection of the immunoblot. Protein molecular weight markers are indicated on the right.